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(54) Title: RECOMBINANT ANTIBODY-ENZYME FUSION PROTEINS

(57) Abstract

The invention relates to recombinantly produced fusion polypeptides comprising antibody V_H and V_L sequences operatively linked to a β-lactamase for use in the delivery of cytotoxic drugs to tumor cells.

RECOMBINANT ANTIBODY-ENZYME FUSION PROTEINS

BACKGROUND OF THE INVENTION

A considerable amount of attention has been directed towards the use of monoclonal antibody-enzyme conjugates in combination with suitable prodrugs for the selective delivery of chemotherapeutic agents to tumors (reviewed in Senter et al., Bioconjugate Chem., 4:3-9 (1993); Jungheim et al., Chem Rev., 94:1553-1566 (1994); Bagshawe et al., Ann. Oncol., 5:879-891 (1994)). The monoclonal antibody (mAb) portions of these immunoconjugates recognize tumor-selective antigens and are capable of delivering the enzymes to tumor masses. Once tumor localization and systemic conjugate clearance has taken place, a non-cytotoxic prodrug form of a chemotherapeutic drug is administered which is converted into an active drug by the targeted enzyme. This leads to the selective delivery of anticancer drugs to sites of neoplasia. Pharmacokinetic studies have shown that the intratumoral drug concentrations resulting from mAb-enzyme/prodrug combinations can be significantly greater than that achieved by systemic drug administration (Bosslet et al., Cancer Res., 54:2151-2159 (1994); Svensson et al., Cancer Res., 55:2357-2365 (1995); Wallace et al., Cancer Res., 54:2719-2327 (1994)). This probably accounts for the observed antitumor activities, which include complete tumor regressions and cures in a number of different models for human cancer (Springer et al., Eur. J. Cancer, 27:1361-1366 (1991); Meyer et al., Cancer Res., 53:3956-3963 (1993); Eccles et al., Cancer Res., 54:5171-5177 (1994); Kerr et al., Cancer Res., 55:3558-3563 (1995)).

Kerr et al. disclosed the use of antibodies against the human p97 (melanotransferrin) tumor antigen for the delivery of β-lactamase (bL) to tumor cells (Kerr et al., Cancer Res., 55:3558-3563 (1995)). This antigen has been found to be overexpressed on a majority of clinical melanoma isolates and is also observed on many human carcinomas

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(Woodbury et al., Proc. Natl. Acad. Sci. (USA), 77:2183-2187
(1980); Brown et al., J. Immun., 127:539-545 (1981), Brown et
al., Proc. Natl. Acad. Sci. (USA), 78:539-543 (1981); Rose et
al., Proc. Natl. Acad. Sci. (USA) (1986)). Significant
5   antitumor activities have been obtained using the combination
of a chemically produced anti p97-Fab'-bL conjugate in
combination with CCM (7-(4-carboxybutan-amido)cephalosporin
mustard; Kerr et al., Cancer Res., 55:3558-3563 (1995)), a
cephalosporin containing prodrug of phenylenediamine mustard
10   (PDM). These effects were observed in a melanoma tumor model
that was resistant to the activities of PDM.

        A major concern in the development of mAb-bL
conjugates for clinical testing is conjugate uniformity.
Typically, mAb-enzyme conjugates are prepared using
15   bifunctional cross-linking reagents that react in a random
fashion with exposed amino acid residues on the individual
proteins. Immunoconjugates produced in this manner are
microscopically heterogeneous due the inherent lack of
specificity of the cross-linking reagents. In addition,
20   chemically prepared conjugates are typically isolated in low
yields. Although recent reports describe alternative coupling
chemistries that can afford higher yields of more homogeneous
immunoconjugates (Mikolajczyk et al., Bioconjugate Chem.,
5:636-646 (1994); Werlen et al., Bioconjugate Chem., 5:411-417
25   (1994); Werlen et al., Tumor Targeting, 1:251-258 (1995)),
these methods still involve chemical modification steps that
can contribute to product heterogeneity.

        Genetically constructed fusion proteins offer an
alternative method of producing homogeneous mAb-enzyme
30   conjugates. There have now been reports describing the
production, characterization, and activities of recombinant
Fab, sFv, and disulfide stabilized Fv-enzyme fusion proteins
(Bosslet et al., Br. J. Cancer, 65:234-238 (1992); Goshorn et
al., Cancer Res., 53:2123-2127 (1993); Rodrigues et al.,
35   Cancer Res., 55:63-70 (1995)).

        Thus, a need exists for homogeneous mAb-enzyme conjugates
for use in combination with prodrugs. The instant invention
addresses this need and more.

SUMMARY OF THE INVENTION

One aspect of the invention is a fusion polypeptide comprising antibody variable light and heavy region amino acid sequences specific for a melanoma associated antigen operatively linked to a β-lactamase.

A further aspect of the invention is a method for the delivery of a cytotoxic agent to a tumor cell comprising the administration of a fusion polypeptide comprising antibody variable light and heavy region sequences operatively linked to a β-lactamase, wherein the fusion polypeptide is reactive with a tumor cell antigen and the fusion polypeptide converts a prodrug to a cytotoxic drug, and the administration of an effective amount of the prodrug.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the structures of the cephalosporin mustard prodrug CCM and the parent drug phenylenediamine mustard PDM.

Fig. 2 depicts Scatchard binding analysis of L49 antibody binding to 3677 melanoma cells.

Fig. 3 depicts the construction of L49-sFv-bL. Three successive hybridization insertion reactions were used to install the 218 linker, variable heavy chain sequences, and variable light chain sequences into a pET phagemid containing the r2-1 mutant of the *E. cloacae* β-lactamase. Single stranded phagemid DNA was produced by infection of XL-I Blue carrying the pET phagemids with M13KO7 helper phage. An oligonucleotide coding for the 218 linker sequence (- strand), with complementary regions to the 3' end of the pelB sequence and the 3' end of the β-lactamase gene was prepared by chemical synthesis. Corresponding V_H and V_L sequences (- strand) were generated by asymmetric PCR.

Figs. 4A and 4B depicts the nucleotide (SEQ ID. NO: 19) and amino acid (SEQ ID. NO: 20) sequence for L49-sFv-bL including the PelB leader sequence.

Figs. 5A and 5B depict SDS-PAGE (4-20%) and isoelectric focusing analyses (pH 3-10) of recombinant sp97 and wild type p97 antigen. Fig. 5A depicts non-reducing and

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reducing SDS-PAGE analysis of recombinant sp97 and p97 antigen.

Figs. 6A-6D depict SDS-PAGE analyses of L49-sFv-bL expression and purification. Fig. 6A depicts the induction of L49-sFv-bL at varying IPTG concentrations (30°C, total cellular protein, 12% tris-glycine SDS-PAGE, Commassie staining, non-reducing conditions). The band corresponding to L49-sFv-bL is indicated with arrows. Fig. 6B depicts a Western analysis with rabbit polyclonal anti-bL. Lane 1: periplasm; Lane 2: L49-sFv-bL standard (12% tris-glycine SDS-PAGE, non-reducing conditions). Fig. 6C depicts the purification of L49-sFv-bL. Lane 1, periplasm; Lane 2, flow through from the sp97 affinity column; Lane 3, material that eluted from the sp97 column at pH 11; Lane 4, material that bound and eluted off the phenylboronic acid column (12% tris-glycine SDS-PAGE, Commassie staining, non-reducing conditions). Fig. 6D is a representative comparison of L49-sFv-bL to chemically prepared L49-Fab'-bL. Lane 1, L49-Fab'-bL; Lane 2, L49-sFv-bL (10% tris-glycine SDS-PAGE, Commassie staining, non-reducing conditions).

Fig. 7 depicts a competition binding assay. 3677 cells were incubated with various combinations of the test samples (L49, L49-sFv-bL, L49-Fab'-bL, and FITC-modified whole L49, keeping the total mAb concentration (test sample + L49-FITC) constant at 400 nM. Fluorescence intensity was determined by fluorescence activated cell sorter analysis.

Fig. 8 depicts cytotoxic effects of mAb-bL + CCM combinations on 3677 melanoma cells as determined by the incorporation of [^{3}H]thymidine into DNA. 3677 cells were incubated with the mAb-bL conjugates, washed, and treated with CCM for 1 h. The effects were compared to cells treated with CCM or PDM for 1 h without prior conjugate exposure and to cells that were treated with saturating, amounts of unconjugated L49 prior to conjugate 1 treatment.

Figs. 9A and 9B depict pharmacokinetics of L49-sFv-bL in nude mice (3 animals/group). L49-sFv-bL was injected intravenously, tissues were removed and extracted at the indicated times, and the β-lactamase activity was determined

using nitrocefin as a substrate. Fig. 9A depicts clearance of
L49-sFv-bL from the blood. Injected dose was 4 mg/kg. Fig.
9B depicts L49-sFv-bL levels in subcutaneous 3677 melanoma
tumors and in normal tissues. Injected dose was 1 mg/kg.

5   Figs. 10A-10D. Therapeutic effects of L49-sFv-bL/CCM
combinations in nude mice (six mice/group) with subcutaneous
3677 melanoma xenografts. Conjugates were injected, followed
at various times by CCM (arrows on the X-axis). The average
tumor volumes were reported until most or all of the animals
10   were cured (tumors that became non-palpable for ≥ 10 tumor
volume doubling times), or until an animal was removed from
the experiment due to tumor outgrowth. Fig. 10A. L49-sFv-bL
(1 mg/kg/injection) 12 h before CCM. Fig. 10B. L49-sFv-bL
(1 mg/kg/injection) 24 h before CCM. Fig. 10C. L49-sFv-bL
15   (4 mg/kg/injection) 24 h before CCM. Fig. 10D. L49-sFv-bL
(4 mg/kg/injection) 48 h before CCM.

                DETAILED DESCRIPTION OF THE INVENTION
            The present invention encompasses pharmaceutical
20   compositions, combinations and methods for treating cancers
and other tumors. More particularly, the invention includes
combinations comprising the antibody-enzyme fusion
polypeptides of the invention and a corresponding prodrug or
prodrugs for use in a method for treating tumors wherein a
25   mammalian host is treated in a pharmaceutically acceptable
manner with a pharmaceutically effective amount of an
antibody-enzyme fusion polypeptide and a pharmaceutically
effective amount of a prodrug or prodrugs. The combination
and methods of this invention are useful in treating any
30   mammal, including humans, dogs, cats, and horses.
            In some embodiments the enzyme component of the
antibody-enzyme fusion is a β-lactamase. The β-lactamase (bL)
can be a class A, B, C, or D enzyme (Ledent et al., Biochem.
J., 292:555-562 (1993); Felici et al., Biochem. J., 291:151-
35   155 (1993)). Preferably, the bL is a class C enzyme. More
preferably, the bL is an Enterobacter cloacae enzyme (Dubus et
al., Biochem. J., 301:485-494 (1994). The enzyme can be wild-
type or mutant. For example, the E. cloacae P99 bL fusion

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protein can be substituted at amino acid residues 537-541 (Gly
Ser Asp Ser Lys (SEQ ID. NO: 1)). Possible substitutions at
this region include Thr Ser Phe Gly Asn (SEQ ID. NO: 2), Ala
Ser Ala Arg Arg (SEQ ID. NO: 3), Asn Asn Ala Gly Tyr (SEQ ID.

5   NO: 4), Glu Val Glu Ile Lys (SEQ ID. NO: 5), Leu Thr Ser Asn
Arg (SEQ ID. NO: 6), Gly Ser Lyn Ser His (SEQ ID. NO: 7), Val
Thy Arg Asn Gln (SEQ ID. NO: 8), Ile Val Asn Asn Lys (SEQ ID.
NO: 9), Thr Ala Ile Pro Asp (SEQ ID. NO: 10) and Ile Thr Lys
Pro Asp (SEQ ID. NO: 11) (Siemers et al., Biochemistry

10   35:2104-2111 (1996). A preferred mutant is Thr Ser Phe Gly
Asn (SEQ ID. NO: 2).

        The antibody component of the fusion can be obtained
from any species. In an embodiment, the antibody is a murine
monoclonal antibody directed against an antigen characteristic

15   of a tumor cell to be targeted. Techniques for generating
such monoclonal antibodies are well known in the art.

        Antibodies can also be obtained from phage display and
bacterial surface display libraries. This includes those
produced from human tissue sources (to make human monoclonal

20   antibodies) and combinatorial libraries of monoclonal
antibodies produced by mutagenesis of CDR loops.

        Typically, nucleic acid sequences encoding the
variable regions of the light chains of the antibody are
cloned by techniques well known in the art, such as by random

25   hexamer primed reverse transcription reactions and PCR with
signal sequence and constant region PCR primers (Jones et al.,
Biotechnology, 9:88-92 (1991)).

        The corresponding DNA sequences are inserted into a
vector of choice. Typically a secretory leader is operatively

30   linked to the V_H and V_L region sequences. Any secretory
leader can be used that will direct the secretion of the
fusion protein into the periplasm of E. coli or other host, or
into the medium. Exemplary secretory leaders include PelB,
OmpA and StIII.

35   Typically the V_H and V_L region coding sequences are
separated by a spacer that is generally greater than or equal
to 15 amino acids. Examples include the sequence Gly Gly Gly
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser (SEQ ID. NO:

12) and the sequence Gly Ser Thr Ser Gly Ser Gly Lys Pro Gly Ser Gly Glu Gly Ser Thr Lys Gly (SEQ ID. NO: 13) (218 linker). The V_L region gene is linked in continuous reading frame with the enzyme.

5   The entire fusion construct can be replicated as part of a vector that can be propagated in a host of interest, including but not limited to bacterial, yeast, insect, and mammalian hosts.

          The prodrugs are preferably cephalosporin derivatives

10   of phenylenediamine mustard, doxorubicin, mitomycin C, paclitaxel, vinca alkaloids, and melphalan. Other anti-cancer agents with active amines, hydroxyl or thiol groups can be modified with cephalosporins to form drugs that are activated by β-lactamases.

15   According to a preferred embodiment, the antibody-enzyme fusion polypeptide is administered prior to the introduction of the prodrug into the host. Sufficient time should be allowed between administration of the conjugate and the prodrug to allow the antibody component of the conjugate

20   to target and localize the enzyme to the tumor site. Such sufficient time may range from 4 hours to one week depending upon the conjugate used.

          The conjugates and prodrugs of the invention can be administered using conventional modes of administration

25   including, but not limited to, intravenous, intraperitoneal, oral, intralymphatic, or administration directly into the tumor. Intravenous administration is preferred.

          The compositions of the invention—comprising the immunoconjugates or prodrugs—may be in a variety of dosage

30   forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic

35   application. For example, oral administration of the antibody-enzyme conjugate may be disfavored because the conjugate proteins tend to be degraded in the stomach if taken orally, e.g., in tablet form.

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The conjugate or prodrug compositions also preferably
include conventional pharmaceutically acceptable carriers and
adjuvants known in the art such as human serum albumin, ion
exchangers, alumnia, lecithin, buffer substances such as
5   phosphates, glycine, sorbic acid, potassium sorbate, and salts
or electrolytes such as protamine sulfate.

The most effective mode of administration and dosage
regimen for the compositions of this invention depends upon
the severity and course of the disease, the patient's health
10   and response to treatment and the judgement of the treating
physician. Accordingly, the dosages of the immunoconjugates
and prodrugs should be titrated to the individual patient.

Nevertheless, an effective dose of the antibody-enzyme
conjugate of this invention may be in the range of from about
15   1.0 to about 100 mg/m^2. An effective dose of the prodrug of
the invention will depend upon the particular prodrug used and
the parent drug from which it is derived. Since the prodrug
is less cytotoxic than the parent drug, dosages in excess of
those recognized in the art for the parent drug may be used.
20   For example, an effective dose of the cephalosporin mustard
prodrugs may be in the range of about 500 mg/m^2.

In order that the invention described herein may be
more fully understood, the following examples are set forth.
It should be understood that these examples are for
25   illustrative purposes only and are not to be construed as
limiting the scope of this invention in any manner.

EXPERIMENTAL EXAMPLES

Materials. The *Enterobacter cloacae* P99 gene was
30   obtained from the plasmid pNU363 (Galleni et al., Biochem. J.,
250:753-760 (1988)). The r2-1 bL was isolated from a library
of *E. cloacae* enzymes in which the nucleotides corresponding
to amino acids 286-290 were subjected to codon-based
mutagenesis (Siemers et al., Biochemistry, 35:2104-2111
35   (1996)), and contains the residues Thr Ser Phe Gly Asn (SEQ
ID. NO: 2) at these positions. L49-Fab'-bL was prepared as
previously described by combining thiolcontaining Fab'
fragments of the antibodies with maleimide-substituted bL,

forming a thioether link between the two proteins (Svensson et al., _Bioconjugate Chem._, 5:262-267 (1994)).

        Isolation and Characterization of the L49 Antibody.
The L49 producing hybridoma was developed using standard
techniques as previously described for the isolation of other
hybridomas (Yeh et al., _Proc. Natl. Acad. Sci. (USA)_, 76:2927-
2931 (1979)). Balb/C mice were immunized repeatedly with the
H2981 (lung carcinoma), CH3 (lung carcinoma), and W56
(melanoma) cell lines, all of which were derived from human
tumors. Spleen cells from the immunized mice were hybridized
with the neomycin gene transfected myeloma cell line P3X63-
Ag8.563 (Yeh et al., _Proc. Natl. Acad. Sci. (USA)_, 76:2927-
2931 (1979)). Standard selection and cloning yielded a
hybridoma producing the L49 IgG1 antibody.

        Scatchard analysis of L49 binding was performed by
radiolabeling the mAb with [^{125}I]Iodogen to a specific
activity of 0.3 mCi/mg protein. 3677 melanoma cells (Kerr et
al., _Cancer Res._, 55:3558-3563 (1995)) in 96-well plates
(13,000 cells/well) were incubated with 0.03 to 10 nM ^{125}I-L49
for 30 min. on ice, and then the cells were separated from
unbound radioactivity by centrifugation through silicon oil.
The tubes were frozen, the cell pellet was cut from the
supernatant, and both fractions were counted in a gamma
counter (Packard). Binding affinity and sites per cell were
determined by Scatchard analysis (Trucco and dePetris,
Immunological Methods, I. Lefkovits and B. Pernis, eds.,
Academic Press, New York, pp. 1-26 (1981)).

        Soluble p97 (sp97). A secreted form of p97 (sp97) was
made utilizing PCR based mutagenesis to introduce a stop codon
at the cysteine residue three amino acids upstream of the
glycophosphatidylinositol anchor domain (Alemany et al., _J.
Cell Sci._, 104:1155-1162 (1993); Food et al., _J. Biol. Chem._,
269:3034-3040 (1994)). Briefly, the 3' oligonucleotide used
in the PCR reaction contained the mutation changing the S710
codon to a stop codon. In all, the coding sequence for 29
amino acids was deleted from the carboxyl terminus of wild
type p97. Cloning and expression of sp97 was accomplished
using a glutamine synthetase gene as an amplifiable marker in

CHO cells (Cockett et al., _Biotechnology_, 8:662-667 (1990)).
The sp97 gene was cloned into pEE14 (Stephens and Cockett,
Nucl. Acids Res., 17:7110 (1989)) and transfected into CHO-K1
cells by calcium phosphate coprecipitation. Transformants

5   were initially selected for resistance to 25 μM methionine
sulfoximine, and sp97 secreting colonies were selected for
amplification at drug concentrations of 100, 250, and 500 μM.
The selection and amplification media used was Glasgow Minimum
Essential Medium without L-glutamine, tryptose phosphate broth

10   or sodium bicarbonate supplemented with 10% dialyzed fetal
bovine serum. A cloned CHO cell line secreting sp97 was
cultured in 10-shelf cell factories containing 1.5 liters of
media.

         Soluble p97 was isolated on a 96.5 immunoaffinity

15   chromatography column as described for the purification of
wild type p97 from melanoma cells (Baker et al., _FEBS Lett._,
298:215-218 (1992)). Small amounts of residual contaminants
were removed by gel filtration on a Sephacryl S300 HR column
(Pharmacia LKB) using PBS as eluant. Solutions containing

20   sp97 were concentrated by ultrafiltration to 1-5 mg/ml,
sterilized by passage through a 0.1 μ filter, and stored at 2-
8°C for up to 6 months without noticeable loss of biochemical
or biological activity.

         Cloning of L49 Variable Regions and sFv Construction.

25   Construction of L49-sFv-bL by hybridization insertion was
performed with materials and protocols from the BioRad M13
mutagenesis kit, except for isolation of single stranded
phagemid template (Qiagen M13 kit, M13KO7 helper phage). The
variable regions of the L49 antibody were cloned from the

30   corresponding hybridoma mRNA by reverse transcription-PCR
(Perkin Elmer GeneAmp reagents and Model 9600 thermal cycler),
using random hexamer primed reverse transcription reactions
and signal sequence and constant region PCR primers (Jones et
al., _Biotechnology_, 9:88-92 (1991)). Construction of L49-sFv-

35   bL began with a single-stranded template of the pET-26b
phagemid containing the r2-1 mutant of the _Enterobacter
cloacae_ P99 bL gene (Siemers et al., _Biochemistry_, 35:2104-
2111 (1996)) fused to the pelB leader sequence. Hybridization

mutagenesis was used to insert the 218 linker sequence
(Whitlow et al., *Protein Eng.*, 6:989-995 (1993)) (chemically
synthesized oligonucleotide, 5'- TTCTGACACTGGCGTGCCCTTGGTA
GAGCCTTCGCCAGAGCCCGGTTTGCCAGAGCCGGACGTCGAGCCGGCCATCGCCGGCTG-3'
(SEQ ID. NO: 14)) and full V_H and V_L region sequences
(oligonucleotides produced by asymmetric PCR, V_H forward
primer: 5'-CCAGCCGGCGATGGCCGAGGTGCAGCTTCAGGAGT-3' (SEQ ID. NO:
15); V_H reverse primer: 5'-AGAGCCGGACGTCGAGCCTGAGGAGACGGT
GACAGAGG-3'(SEQ ID. NO: 16); V_L forward primer: 5'-
AGGCTCTACCAAGGGCGATTTTGTGATGACCCAAAC-3' (SEQ ID. NO: 17); V_L
reverse primer: 5'-TTCTGACACTGGCGTCCGTTTGATTTCCAGCTTGG-3'(SEQ
ID. NO: 18) in between the pelB leader sequence and bL in a
5'-pelB-V_H-218-V_L-bL-3' orientation. The nucleotide (SEQ ID
NO: 19) and amino acid (SEQ ID NO. 20) sequences of L49-sFv-bL
are provided in Figs. 4A and 4B.

    **Expression, Purification and Characterization of L49-
sFv-bL.** L49-sFv-bL was expressed as a soluble protein in *E.
coli* strain BL21 (λDE3) at 23°C in 4L, baffled shake flasks.
T-broth (1L) containing 30 μg/ml kanamycin was inoculated with
several colonies of freshly transformed BL21 (λDE3) cells.
The flasks were shaken (200 rpm) at 37°C until the absorbance
at 660 nm reached 0.8. The culture was cooled to 23°C and IPTG
(50 μM) was added. The culture was incubated with shaking for
an additional 16 h at 23°C, at which time the absorbance at
660 nm was between 8-15. The cells were pelleted by
centrifugation and resuspended in 30 mM Tris, 2 mM EDTA, 0.3%
(v/v) Nonidet P-40, pH 8.5, 4°C. The mixture was stirred
gently for 1 h, repelleted, and the supernatant was decanted
and filtered (0.2 μm).

        Purification of L49-sFv-bL was accomplished by a two-
step affinity purification. The periplasmic fraction was
first applied to a Sepharose column of immobilized sp97
antigen. The column was washed with PBS until the absorbance
at 280 nm reached the baseline level, and bound protein was
eluted with pH 11.2 buffer (50 mM sodium phosphate, 100 mM
NaCl). Fractions containing the bound protein were
neutralized with 1/10 v/v of 3M phosphate, pH 7.2. This
material was then subjected to Sepharose 4B m-

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aminophenylboronic acid affinity chromatography (Cartwright et
al., _Biochem. J._, 221:505-512 (1984)) using washing and
elution conditions described above. The resulting preparation
was dialyzed against PBS, filtered (0.2 μm), and stored at 4°C
5   (0.1-1.1 mg/ml).

Competition binding experiments were performed as
described by Svensson et al.(_Bioconjugate Chem._, 3:176-181
(1992)). Immunoassays were performed by coating polystyrene
96 well plates with sp97 (0.1 mL, 2 μg/ml in PBS, overnight,
10   4°C.) After blocking with specimen diluent (Genetic Systems
Corp.) for 1 h at 22°C, the blocking solution was replaced
with fresh specimen diluent (0.1 ml) containing serial
dilutions of the samples. After 1 h at 22°C the plates were
washed, followed by development with 0.1 mL of a nitrocefin
15   (O'Callaghan et al., _Antimicrobial Agents and Chemotherapy_,
1:283-288 (1972)) solution (0.1 mM in PBS/1%
dimethylformamide, 15·min, 22°C. Absorbance measurements were
read in an plate reader using a 490 nm filter with 630 nm as
the reference wavelength.

20   **In Vitro Cytotoxicity.** 3677 melanoma cells were
plated into 96-well microliter plates (10^4 cells/well in 100
μL of Iscove's Modified Dulbecco's Medium (IMDM) with 10%
fetal bovine serum, penicillin (60 μg/ml), and streptomycin
(100 μg/ml) and allowed to adhere overnight. For blocking
25   experiments, the cells were incubated with unconjugated L49 at
1 μM for 30 min. prior to treatment with the L49 conjugates.
The cells were treated with L49-sFv-bL or L49 Fab'-bL at 10
nM. After 30 min. at 4°C, the plates were washed three times
with antibiotic-free RPMI 1640 media (Gibco) with 10% fetal
30   bovine serum, and then varying concentrations of CCM (Fig. 1)
were added. CCM and PDM were also added to cells treated with
media alone. After 1 h at 37°C, cells were washed three times
with IMDM and incubated approximately 18 h at 37°C. The cells
were then pulsed for 12 h with [^{3}H] thymidine (1 μCi/well) at
35   37°C, detached by freezing at -20°C and thawing, and harvested
onto glass fiber filter mats using a 96-well harvester
(Wallac, Gaithersburg, MD). Radioactivity was counted using a
LKB Wallac β-plate counter.

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In Vitro Cytotoxicity. 3677 melanoma cells were
plated in 96-well microtiter plates (10^4 cells/well in 100 μL
of IMDM with 10% fetal bovine serum, 60 μg/ml penicillin and
0.1 mg/ml streptomycin) and allowed to adhere overnight. For

5   blocking experiments, the cells were incubated with
unconjugated L49 at 1 μM for 30 min prior to treatment with
the L49 conjugates. The cells were treated with L49-sFv-bL or
L49 Fab'-bL at 10 nM. After 30 min at 4°C, the plates were
washed three times with antibiotic-free RPMI 1640 media

10   (Gibco) with 10% fetal bovine serum, and then varying
concentrations of CCM were added. CCM and PDM were also added
to cells treated with media alone. After 1 h at 37°C, cells
were washed three times with IMDM and incubated approximately
18 h at 37°C. The cells were then pulsed for 12 h with [^{3}H]

15   thymidine (1 μCi/well) at 37°C, detached by freezing at -20°C
and thawing, and harvested onto glass fiber filter mats using
a 96-well harvester. Radioactivity was counted using a LKB
Wallac β-plate counter.

Conjugate Localization. Subcutaneous 3677 melanoma
20   tumors were established in female athymic nu/nu mice (8-12
weeks old, Harlan Sprague-Dawley, Indianapolis, IN) by
transplanting tumors that had been previously passaged as
previously described (Kerr et al., _Cancer Res._, 55:3558-3563
(1995)). Tumor bearing mice were injected i.v. with L49-sFv-

25   bL (1 or 4 mg mAb component/kg) or with L49-Fab'-bL (1.8 mg
mAb component/kg). At various time intervals, the mice were
anesthetized, bled through the orbital plexus, and sacrificed.
Tissues were removed and homogenized in PBS containing 15
μg/ml aprotinin (2 mL/g tissue). To the homogenate was added

30   50 mM sodium phosphate containing 100 mM NaCl at pH 11.2 (10
ml/g tissue), and the suspension was mixed. After 20 min at
room temperature, 3M sodium phosphate at pH 7.0 was added (2
ml/g tissue), and the mixture was mixed and centrifuged.

Quantification of conjugate concentrations was
35   accomplished using a direct enzyme immunoassay. Polystyrene
96-well microtiter plates were coated with an affinity-
purified rabbit polyclonal antisera to wild type _E. cloacae_ bL
(1 μg/ml), and were then blocked with specimen diluent

(Genetic Systems Corp.). Serially diluted tissue extracts or
purified samples (L49-sFv-bL as a standard for the fusion
protein samples, L49-Fab'-bL as a standard for the L49-Fab'-bL
samples) were added to the wells and allowed to bind for 3 h

5   at room temperature. The plates were washed and developed by
the addition of 0.1 ml of nitrocefin (O'Callaghan et al.,
<u>Antimicrobial Agents and Chemotherapy</u>, 1:283-288 (1972)) at
0.1 mM in PBS containing 1% dimethylformamide. Absorbance
measurements were read in an ELISA plate reader using a 490 nm

10   filter with 630 nm as the reference wavelength.

        In Vivo Therapy Experiments. 3677 tumor-bearing mice
(subcutaneous implants, six animals/group, average tumor
volume 130 mm^3) were injected with L49-sFv-bL (i.v., 7-8 days
post tumor implant), followed 12-48 h later by CCM using doses

15   of fusion protein and prodrug as indicated in the Results
section. Treatment with L49-sFv-bL + CCM was repeated 1 week
later. Animals were monitored 1-2 times/week for body weight,
general health, and tumor growth. Tumor volume was estimated
using the formula: longest length X perpendicular

20   dimension^2/2. Cures were defined as an established tumor
that, after treatment, was not palpable for ≥ 10 tumor volume
doubling delays (≥ 40 days in the 3677 tumor model). Maximum
tolerated doses led to less than 20% weight loss, no
treatment-related deaths, and were within 50% of the dose

25   where such events took place.

RESULTS

        Characterization of the L49 Antibody. The L49
antibody (IgG1) binds to the p97 antigen, which has been shown

30   to be present on most human melanomas and many carcinomas
(Brown et al., <u>J. Immun.</u>, 127:539-545 (1981), Brown et al.,
<u>Proc. Natl. Acad. Sci. (USA)</u>, 78:539-543 (1981); Woodbury et
al., <u>Proc. Natl. Acad. Sci. (USA)</u>, 77:2183-2187 (1980)).
Scatchard analysis of the binding of radiolabeled L49 to the

35   3677 human melanoma cell line indicated that the mAb bound
with a dissociation constant of 1.0 nM (Fig. 2). At
saturation, there were approximately 2.1 x 10^4 molecules of
L49 bound/cell. These values are very similar to those

obtained for the 96.5 mAb (Kerr et al., <u>Cancer Res.</u> 55:3558-
3563 (1995)), which also binds to p97, but to a different
epitope than L49 (data not shown).

   Cloning and Expression of L49-sFv-bL. The variable
5 region genes for the L49 antibody heavy and light chains were
cloned from the L49 hybridoma line by RT-PCR of hybridoma mRNA
and amplification of the corresponding cDNA. A consensus
sequence was determined by examining several clones from
independent reverse transcription reactions to reduce the
10 possibility of reverse transcription or PCR derived errors.
The PCR primers used were complementary to the signal sequence
and constant region of the mAb. Thus, the entire variable
regions were obtained.

   L49-sFv-bL was constructed in a stepwise fashion by
15 hybridization insertion of the sFv linker, V_H, and V_L region
sequences onto a single stranded pET phagemid template
containing the pelB leader sequence and bL gene (Fig. 3). The
particular bL gene used encoded a mutated form of bL (r2-1)
that contained the sequence Thr Ser Phe Gly Asn (SEQ ID. NO:
20 2) at positions 286-290. This mutated bL has been shown to
have slightly greater activity than the wild type enzyme
(Siemers et al., <u>Biochemistry</u>, 35:2104-2111 (1996)). The 218
linker sequence corresponds to amino acids Gly Ser Thr Ser Gly
Ser Gly Lys Pro Gly Ser Gly Glu Gly Ser Thr Lys Gly (SEQ ID.
25 NO: 13), and was used as the sFv linker based on its ability
to reduce sFv protein aggregation (Whitlow et al., <u>Protein
Eng.</u>, 6:989-995 (1993)). An oligonucleotide coding for the
218 linker (- strand, produced by chemical synthesis) was
first annealed to the phagemid template, resulting in a pelB-
30 218-bL construct. V_H and V_L region segments (produced by
asymmetric PCR) were then inserted into the intermediate
construct to generate the final L49-sFv-bL gene in an pelB-V_H-
218-V_L-bL orientation. The pelB leader sequence results in
transport of the protein into the periplasmic space of <i>E.
35 coli.</i> No additional linker was placed between V_L and the bL
enzyme. The nucleotide (SEQ ID NO: 19) and amino acid (SEQ ID
NO: 20) sequences are provided in Figs. 4A and 4B.

        To facilitate the isolation and characterization of
L49-containing fusion proteins, a soluble form of the p97
antigen was developed. This was made by truncating the p97
antigen at a site upstream to the membrane anchoring domain.

5   The soluble antigen (sp97) was expressed in CHO-K1 cells and
purified by affinity chromatography. SDS-PAGE analysis of
recombinant sp97 indicated that it was slightly lower in
molecular weight than p97 (Fig. 5A). Isoelectric focussing
revealed little difference between p97 and sp97 (Fig. 5B), a

10 result that was anticipated, since only a single charged
residue is lost in the sp97 construct.

        L49-sFv-bL was expressed in soluble form in an _E. coli_
strain that was transformed with the plasmid shown in Fig. 3.
Quantitation of L49-sFv-bL containing samples was performed

15 using an immunoassay in which the L49 portion was captured
onto microtiter plates that were coated with sp97, and the bL
enzyme activity was determined using nitrocefin as a
calorimetric indicator for bL activity (O'Callaghan et al.,
Antimicrobial Agents and Chemotherapy, 1:283-288 (1972)).

20 Thus, only bifunctional fusion protein was measured. Under
the transcriptional control of the T7 promoter and lac operon,
fusion protein expression could be detected by SDS-PAGE
analyses of cell pellets when at IPTG concentrations as low as
1.6 μM (Fig. 6A). Significant levels of toxicity were

25 observed when the IPTG concentration exceeded 90 μM, resulting
in inhibition of cell growth and in the eventual outgrowth of
cell populations that did not express fusion protein.
Typically, 50μM IPTG induction was used for large scale
experiments, since this led to higher levels of fusion protein

30 expression without significant levels of cytotoxicity. It was
also found that expression of L49-sFv-bL was enhanced at lower
temperatures, such that protein yields were higher at 23 or
30°C compared to 37°C. Similar results have been noted for
the expression of antibody fragments and other recombinant

35 proteins in _E. coli_ (Plückthun, _Immun. Rev._, 130:151-188
(1992)).

        In shake flask cultures, 80% of active material was
present in the periplasm of the bacterial cells, with the

remainder present in the culture supernatant. Conventional
techniques for releasing the periplasmic contents, such as
sucrose/lysozyme spheroplasting or osmotic shock, resulted in
only a limited release of the available protein. Similar
results were also obtained using 20% alone. Freeze-thawing or
sonication of cells to release total cytoplasmic material did
not result in an increased yield of functional fusion protein.
It was found that a high yield of fusion protein could be
obtained by treating cell pellets with the detergent Nonidet-
P-40. Expression levels of L49-sFv-bL using this detergent
ranged from 2.5-8 mg/L culture. Western analysis (Fig. 6B)
with a rabbit polyclonal antisera raised to bL showed that
most of the bL containing protein in the preparation was
approximately 63 kDa in molecular weight (theoretical
molecular weight 66.5 kDa). Small amounts of truncated
fragments and aggregated material were also detected.

        Purification of L49-sFv-bL. The purification of L49-
sFv-bL to homogeneity was achieved by a two-step affinity
chromatography procedure. Periplasmic preparations from shake
flask cultures were first applied to an immobilized sp97
affinity column that was capable of binding to the L49 portion
of the conjugate. After extensive washing, bound material was
eluted at pH 11.2 (Fig. 6C). Acidic pH conditions (pH 2.2)
successfully eluted the fusion protein but caused
precipitation of material when working with multi-milligram
quantities of fusion protein. The sp97 chromatography
purified material was approximately 70% pure by size-exclusion
HPLC and SDS-PAGE, with the contaminants consisting of two
bands of approximately 33 kDa molecular weight. The second
step of the purification involved binding the material to
immobilized phenylboronic acid. This resin has previously
been found to bind to β-lactamases, presumably to the active
site of the enzymes (Cartwright and Waley, <u>Biochem. J.</u>
221;505-512 (1984)). This led to the recovery of protein that
was pure by SDS-PAGE analysis (Figs. 6C and 6D).

        L49-sFv-bL Characterization and Activity. In view of
the detergent based release of L49-sFv-bL from the bacteria,
it was important to demonstrate that isolated fusion protein

had been correctly processed and transported into the
periplasm, such that the pelB leader sequence was cleaved from
the amino terminus of the V_H region. This was determined by
subjecting the purified fusion protein to amino acid sequence

5   analysis. The sequence obtained (Glu Val Leu Gln Leu Glu Ser
(SEQ ID. NO: 21)) was identical to the expected V_H amino
terminal sequence, indicating that the leader sequence was
proteolytically clipped, as designed.

The binding characteristics of the sFv portion of the

10   fusion protein were determined using a fluorescent activated
cell sorting competition assay in which fusion protein and
FITC-modified whole L49 competed for binding to cell-surface
antigens on SK-MEL 28 melanoma cells. L49-sFv-bL and the L49
Fab'-bL chemical conjugates bound equally well to the cell

15   line, indicating that the binding activity of the antibody
portion of the conjugate was preserved (Fig. 7). More
detailed information about binding was obtained using surface
plasmon resonance, which allowed the measurement of the on and
off rates of L49-sFv-bL binding to the p97 antigen immobilized

20   on a gold surface (Table 1). This assay established that the
binding affinity of the fusion protein to the p97 antigen (K_d
= 1.0 nM) was comparable to L49 Fab' (K_d = 0.73 nM), and
chemically produced L49-Fab'-bL conjugate (K_d = 1.3 nM).

Table 1.   Binding and enzyme kinetic parameters of L49 and bL containing proteins

Sample	$k_{on}(M^{-1} \cdot s^{-1})$	$k_{off}(s^{-1})$	$k_D(nM)$	$k_{cat}(s^{-1})$	$k_m(\mu M)$
L49 Fab'	2.3×10^5	1.7×10^{-4}	0.73	n.a.[b]	n.a.
r2-1 bL[c]	n.a.	n.a.	n.a.	261	19
L49-Fab'-bL[d]	1.8×10^5	2.4×10^{-4}	1.3	n.d.[c]	n.d.
L49-sFv'-bL[c]	4.1×10^5	4.2×10^{-4}	1.0	232	19

a Values shown are the average of a minimum of two independent experiments, except for L49-Fab'-bL (binding experiment performed once). The range of values obtained in Michaelis-Menten kinetic analyses were within 5% of the means.

b Not Applicable.

c The r2-1 bL contains mutations at positions 286-290 compared to the wild type enzyme (Siemers et al., Biochemistry 35:2104-2111 (1996)).

d Chemically prepared conjugate containing the wild type enzyme.

e Not determined.

Enzymatic activity assays of the bL portion of L49-
sFv-bL were undertaken using nitrocefin as the enzyme
substrate (Table 1). Michaelis-Menten kinetic analyses
confirmed that the fusion protein retained the full enzymatic
5   activity of the mutant bL, enzyme from which it was derived
(Siemers. et al., Biochemistry, 35:2104-2111 (1996)). Thus,
both the binding of the L49 antibody and enzymatic activity of
the E. cloacae r2-1 bL were preserved in the fusion protein.

The cytotoxic effects of L49-sFv-bL in combination
10   with CCM were determined on ·3677 human melanoma cells, which
express the p97 antigen. The experiments were performed by
exposing the cells to the conjugates, and washing off unbound
material before adding various concentrations of CCM. [^{3}H]-
Thymidine incorporation was used to measure cytotoxic
15   activity. The prodrug CCM (IC_{50}, 16 μM) was approximately 50-
fold less toxic to 3677 cells than PDM (IC_{50}, 0.3 μM). As
expected, L49-sFv-bL and L49-Fab'-bL were equally effective at
prodrug activation, and the combinations were equivalent in
activity to PDM (Fig. 8). This indicates that prodrug
20   conversion was efficient under the conditions tested. In
addition, it was found that activation was immunologically
specific, since L49-sFv-bL did not activate CCM on cells that
were previously saturated with L49 mAb before being exposed to
the fusion protein.

25   In Vivo Localization. Biodistribution studies of L49-
sFv-bL and L49-Fab'-bL were carried out in nude mice bearing
subcutaneous (s.c.) 3677 melanoma tumor xenografts. The
conjugates were injected i.v., and at various time points
tissues were removed and extracted under alkaline conditions
30   to disrupt antigen-antibody interactions. The samples were
then trapped a polyclonal antiserum to bL, and bL activity was
measured using nitrocefin as a colorimetric indicator
(O'Callaghan et al, Antimicrobial Agents and Chemotherapy,
1:283-288 (1972)). Control experiments in which L49-sFv-bL
35   was directly injected into excised tumors and tissues
indicated that this extraction procedure recovered 90% of the
injected bL activity.

L49-sFv-bL cleared very rapidly from the blood (Fig. 9A). The initial and terminal clearance half lives ($t_{1/2}\alpha$ and $t_{1/2}\beta$) were 0.3 and 2.5 h, respectively, leading to a 10^4 reduction of L49-sFv-bL blood levels within 24 h of conjugate administration. In spite of this rapid clearance, relatively high intratumoral levels of L49-sFv-bL were measured compared to normal tissues, and the ratio remained high for 24 h (Fig. 9B). At 4 h post L49-sFv-bL administration, the tumor to blood ratio was 13:1. The ratio increased substantially with time, and was 105:1 within 24 h of conjugate administration (Table 2).

Table 2.   Tissue Distribution of Immunoconjugates

Treatment[a]	Time	Tumor	% Injected dose/g (standard deviation)				
			Liver	Spleen	Kidney	Blood	
L49-sFv-bL, 1 mg/kg *tumor/tissue ratios*	4 hrs	1.1 (0.2) *1*	0.021 (0.002) *52*	0.014 (0.008) *79*	0.027 (0.015) *41*	0.084 (0.04) *13*	
L49-sFv-bL, 1 mg/kg *tumor/tissue ratios*	12 hrs	0.53 (0.17) *1*	<0.003 *>177*	<0.003 *>177*	<0.003 *>177*	0.008 (0.001) *66*	
L49-sFv-bL, 1 mg/kg *tumor/tissue ratios*	24 hrs	0.21 (0.01) *1*	<0.003 *>70*	<0.003 *>70*	<0.003 *>70*	0.002 (0.001) *105*	
L49-sFv-bL, 4 mg/kg *tumor/tissue ratios*	12 hrs	0.73 (0.02) *1*	<0.003 *>240*	<0.003 *>240*	<0.003 *>240*	0.009 (0.001) *81*	
L49-sFv-bL, 4 mg/kg *tumor/tissue ratios*	24 hrs	0.29 (0.05) *1*	<0.001 *>290*	<0.001 *>290*	<0.001 *>290*	0.002 (0.0002) *141*	
L49-sFv-bL, 4 mg/kg *tumor/tissue ratios*	48 hrs	0.15 (0.07) *1*	n.d.[b]	n.d.	n.d.	0.001 (0.0002) *150*	
L49-Fab'-bL, 1.8 mg/kg *tumor/tissue ratios*	72 hrs	0.28 (0.26) *1*	0.015 (0.003) *19*	0.010 (0.006) *28*	0.016 (0.005) *18*	0.05 (0.015) *5.6*	

a   Mice (3 animals/group) were injected with conjugates, and at the times indicated, tissues were excised and extracted to remove the conjugate. The percent injected dose was based on the measured bL activity compared to standard curves obtained from extracted tissues that were spiked with known amounts of L49-sFv-bL or L49-Fab'-bL.

b   Not determined.

23

Similar results were obtained using L49-sFv-bL doses of 4
mg/kg. At this dose, very high tumor to blood ratios (141-
151:1) were measured 24-48 h after the conjugate was
administered. Interestingly, chemically produced L49-Fab'-bL
5   cleared quite slowly from the blood and had only a 5.6:1 tumor
to blood ratio 72 h after administration. Thus, L49-sFv-bL
localizes in tumors, clears rapidly from the systemic
circulation, and has significantly improved pharmacokinetic
properties compared to the chemically produced L49-Fab'-bL
10   conjugate.

      Therapeutic Activity. *In vivo* therapy experiments
were performed using the L49-sFv-bL/CCM combination in nude
mice with established s.c. 3677 tumors. This particular tumor
model has previously been shown to be resistant to treatment
15   with doxorubicin, PDM, and CCM (Kerr et al., <u>Cancer Res.</u>,
55:3558-3563 (1995)). In the experiments reported here,
conjugate treatment was initiated 7-8 days after tumor
implant, at which time the tumors were approximately 130 mm^3
in volume. CCM was then administered 12, 24, or 48 h later,
20   and the treatment protocol was repeated after 1 week. Maximum
tolerated doses are defined as those that led to less than 20%
weight loss, no treatment-related deaths, and were within 50%
of the dose where such events took place. A tumor was
considered as having been cured once it was not palpable for
25   at least 10 tumor volume doubling times, based on the tumor
growth of untreated animals (tumor volume doubling time was 4
days). If an animal was removed from the experiment because
of tumor growth, the data from the entire group was no longer
plotted, but the remaining animals were followed for tumor
30   size and general health.

      The maximum tolerated doses of CCM (300
mg/kg/injection) and PDM (3 mg/kg/injection) when administered
weekly for three rounds induced 2 and 6 day delays in tumor
outgrowth, respectively (data not shown). In contrast,
35   pronounced antitumor activity was obtained in mice that
received L49-sFv-bL prior to treatment with CCM (Fig. 10A).
Therapeutic efficacy was schedule and dose dependent. Tumor
cures were obtained in all of the animals that received CCM

(125 and 175 mg/kg/injection) 12 h after treatment with L49-
sFv-bL (Fig. 10A). In this dosing schedule, significant
antitumor activity including four cures in the group of six
mice was obtained when the CCM dose was reduced to 75
5   mg/kg/injection. The remaining two animals in this group had
tumors that underwent partial regressions, but eventually
began to grow after the last prodrug treatment. There were no
apparent toxicities in any of these treatment groups.

        Significant antitumor activity could also be achieved
10   when the prodrug was administered 24 h post conjugate
administration, either by increasing the prodrug dose and
keeping the conjugate dose constant at 1 mg/kg/injection (Fig.
10B), or by increasing the conjugate dose to 4 mg/kg/injection
(Fig. 10C). In both cases, the majority of tumors were cured,
15   again with no evidence of toxicity. Finally, therapeutic
efficacy was also obtained with a 48 h interval between
conjugate and prodrug administration (Fig. 10D). Tumor
regressions were obtained in all of the mice in these groups,
and three of six animals that received 275 mg/kg/injection CCM
20   were cured. Thus, the antitumor activities of L49-sFv-bL in
combination with CCM were pronounced, and therapeutic efficacy
was achieved in a variety of dosing schedules.

        In summary, the fusion protein L49-sFv-bL was
constructed and expressed in *E. coli* as a soluble protein, and
25   isolated from the periplasmic space using a two-stage affinity
chromatography method. After purification, L49-sFv-bL was
demonstrated to be homogeneous by SDS-PAGE, and was fully
active with respect to both the L49 and bL components. As
expected, the fusion protein was able to bind to melanoma
30   cells that expressed the p97 antigen, and were able to
activate a cephalosporin mustard in an immunologically
specific manner.

        To minimize systemic, non-targeted drug release *in
vivo*, a high mAb-enzyme tumor to normal tissue ratio is needed
35   before the anticancer prodrug is administered. To attain the
required localization index in mice, the time between
conjugate and prodrug administration has varied significantly
from one conjugate to another. For example, the delay between

conjugate and prodrug administration was 3 days for 96.5-Fab'-
bL (molecular weight 92 kDa, Kerr et al., Cancer Res.,
55:3558-3563 (1995)), 1 week for the anti CEA-Fab-β-
glucuronidase fusion protein (molecular weight 250 kDa,
5   Bosslet et al., Cancer Res., 54:2151-2159 (1994)), and 2 weeks
for the ICR12-carboxypeptidase G2 conjugate (molecular weight
range of 233-316 kDa, Eccles et al., Cancer Res., 54:5171-5177
(1994)). In some cases, it has even been necessary to
accelerate systemic conjugate clearance in a separate step
10   involving the formation of immune complexes before prodrug
could be administered (Wallace et al., Cancer Res., 54:2719-
2723 (1994); Kerr et al., Bioconjugate Chem., 4:353-357
(1993); Rogers et al., Br. J. Cancer, 72:1357-1363 (1995)).
Here L49-sFv-bL not only clears very rapidly from the systemic
15   circulation (Fig. 9A), but also preferentially localized into
subcutaneous tumor xenografts (Fig. 9B, Table 2). The very
high tumor to non-tumor fusion protein ratios obtained within
4-12 hours of conjugate treatment would lead to the prediction
that, in contrast to other enzyme/prodrug systems (Bosslet et
20   al., Cancer Res., 54:2151-2159 (1994); Eccles et al., Cancer
Res., 54:5171-5177 (1994); Kerr et al., Cancer Res., 55:3558-
3563 (1995)), therapeutic efficacy would not require
protracted time intervals between conjugate and prodrug
administration. This has now been experimentally confirmed,
25   since cures of established tumors were obtained when CCM was
administered 12 h following the conjugate (Fig. 10A).

       It is noteworthy that a correlation can be made
between the outcome in the therapy experiments (Figs. 10A-D)
and the pharmacokinetic data (Figs. 9A and B and Table 2). At
30   a given conjugate dose, the intratumoral concentration
decreased with a half life of approximately 8 h (Table 2).
This may be due to a variety of factors such as dissociation
of the conjugate from the antigen, membrane recycling, enzyme
metabolism, and rapid tumor growth. The net result is that
35   longer time intervals between conjugate and prodrug
administration require that either the amount of prodrug or
conjugate be increased in order to maintain therapeutic
efficacy (Figs. 10A-D).

All references cited herein are specifically incorporated by reference in their entirety for all purposes.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of
5   clarity of understanding, those with ordinary skill in the art will appreciate that other embodiments and variations of the invention are possible which employ the same inventive concepts described above. Therefore, the invention is not to be limited by the above disclosure, but is to be determined in
10   scope by the claims which follow.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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        (I) TELEX:

    (ii) TITLE OF INVENTION: RECOMBINANT ANTIBODY-ENZYME FUSION PROTEINS

    (iii) NUMBER OF SEQUENCES: 21

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Gly Ser Asp Ser Lys
    1   5

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Ser Phe Gly Asn
1   5

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ser Ala Arg Arg
1   5

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Asn Ala Gly Tyr
1   5

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu Val Glu Ile Lys
1   5

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Thr Ser Asn Arg
1   5

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

    Gly Ser Lys Ser His
    1   5

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

    Val Thr Arg Asn Gln
    1   5

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Ile Val Asn Asn Lys
    1   5

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

    Thr Ala Ile Pro Asp
    1   5

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid

          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

     Ile Thr Lys Pro Asp
     1   5

(2) INFORMATION FOR SEQ ID NO:12:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 15 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (v) FRAGMENT TYPE: internal

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

     Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
     1   5   10   15

(2) INFORMATION FOR SEQ ID NO:13:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 16 amino acids
          (B) TYPE: amino acid
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (v) FRAGMENT TYPE: internal

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

     Gly Ser Thr Ser Gly Ser Gly Lys Gly Ser Gly Gly Ser Thr Lys Gly
     1   5   10   15

(2) INFORMATION FOR SEQ ID NO:14:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 84 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTCTGACACT GGCGTGCCCT TGGTAGAGCC TTCGCCAGAG CCCGGTTTGC CAGAGCCGGA   60

CGTCGAGCCG GCCATCGCCG GCTG   84

(2) INFORMATION FOR SEQ ID NO:15:

     (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 35 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CCAGCCGGCG ATGGCCGAGG TGCAGCTTCA GGAGT   35

(2) INFORMATION FOR SEQ ID NO:16:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 38 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AGAGCCGGAC GTCGAGCCTG AGGAGACGGT GACAGAGG   38

(2) INFORMATION FOR SEQ ID NO:17:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 36 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGGCTCTACC AAGGGCGATT TTGTGATGAC CCAAAC   36

(2) INFORMATION FOR SEQ ID NO:18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 35 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTCTGACACT GGCGTCCGTT TGATTTCCAG CTTGG   35

(2) INFORMATION FOR SEQ ID NO:19:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 2010 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
AATTAATACG ACTCACTATA GGGGAATTGT GAGCGGATAA CAATTCCCCT CTAGAAATAA        60

TTTTGTTTAA CTTTAAGAAG GAGATATACA TATGAAATAC CTGCTGCCGA CCGCTGCTGC       120

TGGTCTGCTG CTCCTCGCTG CCCAGCCGGC GATGGCCGAG GTGCAGCTTC AGGAGTCAGG       180

ACCTAGCCTC GTGAAACCTT CTCAGACTCT GTCCCTCACC TGTTCTGTCA CTGGCGACTC       240

CATCACCAGT GGTTACTGGA ACTGGATCCG GAAGTTCCCA GGGAATAAAC TTGAATATAT       300

GGGTTACATA AGCGACAGTG GTATCACTTA CTACAATCCA TCTCTCAAAA GTCGCATTTC       360

CATCACTCGA GACACATCCA GAACCAATA CTACCTCCAG TTGAATTTTG TGACTGCTGA       420

GGACACAGCC ACATATAACT GTGCAAGAAG GACTCTGGCT ACTTACTATG CTATGGACTA       480

CTGGGGTCAA GGAACCTCTG TCACCGTCTC CTCAGGCTCG ACGTCCGGCT CTGGCAAACC       540

GGGCTCTGGC GAAGGCTCTA CCAAGGGCGA TTTTGTGATG ACCCAAACTC CACTCTCCCT       600

GCCTGTCAGT CTTGGAGATC AAGCCTCCAT CTCTTGCAGG GCTAGTCAGA GCCTTGTACA       660

CAGTAATGGA AACACCTATT TACATTGGTA CCTGCAGAAG CCAGGCCAGT CTCCAAAGCT       720

CCTGATCTAC AGAGTTTCCA ACCGATTTTC TGGGGTCCCA GACAGGTTCA GTGGCAGTGG       780

ATCAGGGACA GATTTCACAC TCAAGATCAG CAGAGTGGAG GCTGAGGATC TGGGAGTTTA       840

TTTCTGCTCT CAAAGTACAC ATGTTCCTCC GACGTTCGGT GGAGGCACCA AGCTGGAAAT       900

CAAACGGACG CCAGTGTCAG AAAAACAGCT GGCGGAGGTG GTCGCGAATA CGATTACCCC       960

GCTGATGAAA GCCCAGTCTG TTCCAGGCAT GGCGGTGGCC GTTATTTATC AGGGAAAACC      1020

GCACTATTAC ACATTTGGCA AGGCCGATAT CGCGGCGAAT AAACCCGTTA CGCCTCAGAC      1080

CCTGTTCGAG CTGGGTTCTA TAAGTAAAAC CTTCACCGGC GTTTTAGGTG GGGATGCCAT      1140

TGCTCGCGGT GAAATTTCGC TGGACGATGC GGTGACCAGA TACTGGCCAC AGCTGACGGG      1200

CAAGCAGTGG CAGGGTATTC GTATGCTGGA TCTCGCCACC TACACCGCTG GCGGCCTGCC      1260

GCTACAGGTA CCGGATGAGG TCACGGATAA CGCCTCCCTG CTGCGCTTTT ATCAAAACTG      1320

GCAGCCGCAG TGGAAGCCTG GCACAACGCG TCTTTACGCC AACGCCAGCA TCGGTCTTTT      1380

TGGTGCGCTG GCGGTCAAAC CTTCTGGCAT GCCCTATGAG CAGGCCATGA CGACGCGGGT      1440

CCTTAAGCCG CTCAAGCTGG ACCATACCTG GATTAACGTG CCGAAAGCGG AAGAGGCGCA      1500

TTACGCCTGG GGCTATCGTG ACGGTAAAGC GGTGCGCGTT TCGCCGGGTA TGCTGGATGC      1560

ACAAGCCTAT GGCGTGAAAA CCAACGTGCA GGATATGGCG AACTGGGTCA TGGCAAACAT      1620

GGCGCCGGAG AACGTTGCTG ATGCCTCACT TAAGCAGGGC ATCGCGCTGG CGCAGTCGCG      1680

CTACTGGCGT ATCGGGTCAA TGTATCAGGG TCTGGGCTGG GAGATGCTCA ACTGGCCCGT      1740

GGAGGCCAAC ACGGTGGTCG AGACGAGTTT TGGTAATGTA GCACTGGCGC CGTTGCCCGT      1800

GGCAGAAGTG AATCCACCGG CTCCCCCGGT CAAAGCGTCC TGGGTCCATA AAACGGGCTC      1860

TACTGGCGGG TTTGGCAGCT ACGTGGCCTT TATTCCTGAA AAGCAGATCG GTATTGTGAT      1920
```

GCTCGCGAAT ACAAGCTATC CGAACCCGGC ACGCGTTGAG GCGGCATACC ATATCCTCGA   1980

GGCGCTACAG TAGACTAGTG AATTCGAGCT   2010

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 442 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Met Lys Tyr Thr Ala Ala Ala Gly Ala Ala Ala Met Ala Val Ser Gly
1               5                  10                  15

Ser Val Lys Ser Thr Ser Thr Cys Ser Val Thr Gly Asp Ser Thr Ser
            20                  25                  30

Gly Tyr Trp Asn Trp Arg Lys Gly Asn Lys Tyr Met Gly Tyr Ser Asp
            35                  40                  45

Ser Gly Thr Tyr Tyr Asn Ser Lys Ser Arg Ser Thr Arg Asp Thr Ser
    50                  55                  60

Lys Asn Tyr Tyr Asn Val Thr Ala Asp Thr Ala Thr Tyr Asn Cys Ala
65                  70                  75                  80

Arg Arg Thr Ala Thr Tyr Tyr Ala Met Asp Tyr Trp Gly Gly Thr Ser
            85                  90                  95

Val Thr Val Ser Ser Gly Ser Thr Ser Gly Ser Gly Lys Gly Ser Gly
            100                 105                 110

Gly Ser Thr Lys Gly Asp Val Met Thr Thr Ser Val Ser Gly Asp Ala
            115                 120                 125

Ser Ser Cys Arg Ala Ser Ser Val His Ser Asn Gly Asn Thr Tyr His
    130                 135                 140

Trp Tyr Lys Gly Ser Lys Tyr Arg Val Ser Asn Arg Ser Gly Val Asp
145                 150                 155                 160

Arg Ser Gly Ser Gly Ser Gly Thr Asp Thr Lys Ser Arg Val Ala Asp
                165                 170                 175

Gly Val Tyr Cys Ser Ser Thr His Val Thr Gly Gly Gly Thr Lys Lys
            180                 185                 190

Arg Thr Val Ser Lys Ala Val Val Ala Asn Thr Thr Met Lys Ala Ser
            195                 200                 205

Val Gly Met Ala Val Ala Val Tyr Gly Lys His Tyr Tyr Thr Gly Lys
    210                 215                 220

Ala Asp Ala Ala Asn Lys Val Thr Thr Gly Ser Ser Lys Thr Thr Gly
225                 230                 235                 240

Val Gly Gly Asp Ala Ala Arg Gly Ser Asp Asp Ala Val Thr Arg Tyr
                245                 250                 255

Trp Thr Gly Lys Trp Gly Arg Met Asp Ala Thr Tyr Thr Ala Gly Gly
            260                 265                 270

Val Asp Val Thr Asp Asn Ala Ser Arg Tyr Asn Trp Trp Lys Gly Thr
```

34

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           275                    280                    285

Thr Arg Tyr Ala Asn Ala Ser Gly Gly Ala Ala Val Lys Ser Gly Met
    290             295             300

Tyr Ala Met Thr Thr Arg Val Lys Lys Asp His Thr Trp Asn Val Lys
305             310             315                 320

Ala Ala His Tyr Ala Trp Gly Tyr Arg Asp Gly Lys Ala Val Arg Val
            325             330                 335

Ser Gly Met Asp Ala Ala Tyr Gly Val Lys Thr Asn Val Asp Met Ala
            340             345             350

Asn Trp Val Met Ala Asn Met Ala Asn Val Ala Asp Ala Ser Lys Gly
        355             360             365

Ala Ala Ser Arg Tyr Trp Arg Gly Ser Met Tyr Gly Gly Trp Met Asn
    370             375             380

Trp Val Ala Asn Thr Val Val Thr Ser Gly Asn Val Ala Ala Val Ala
385             390             395                 400

Val Asn Ala Val Lys Ala Ser Trp Val His Lys Thr Gly Ser Thr Gly
            405             410                 415

Gly Gly Ser Tyr Val Ala Lys Gly Val Met Ala Asn Thr Ser Tyr Asn
            420             425             430

Ala Arg Val Ala Ala Tyr His Ala Thr Ser
            435             440
```

(2) INFORMATION FOR SEQ ID NO:21:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 7 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Glu Val Leu Gln Leu Glu Ser
1               5
```

35

WHAT IS CLAIMED IS:

　　　　1.　A fusion polypeptide comprising antibody variable
light and heavy region amino acid sequences specific for a
melanoma associated antigen operatively linked to a β-
lactamase.

　　　　2.　The fusion polypeptide of claim 1, wherein the
fusion polypeptide is operatively linked to a secretory leader
sequence.

　　　　3.　The fusion polypeptide of claim 2, wherein the
secretory leader sequence is pel B.

　　　　4.　The fusion polypeptide of claim 1, wherein the β-
lactamase is *E. cloacae* P99 having one or more amino acid
substitutions at amino acid residues 537-541.

　　　　5.　The fusion polypeptide of claim 4, wherein the
substitution is Thr Ser Phen Gly Asn (SEQ ID. NO: 2) .

　　　　6.　The fusion polypeptide of claim 1, wherein the
variable light and heavy region amino acid sequences are
separated by a spacer.

　　　　7.　The fusion polypeptide of claim 6, wherein the
spacer is Gly Ser Thr Ser Gly Ser Gly Lys Pro Gly Ser Gly Glu
Gly Ser Thr Lys Gly (SEQ ID. NO: 13).

　　　　8.　The fusion polypeptide of claim 1, wherein the
polypeptide specifically binds a tumor cell antigen.

　　　　9.　The fusion polypeptide of claim 8, wherein the
tumor cell antigen is p97.

　　　　10.　The fusion polypeptide of claim 1, wherein the
fusion polypeptide has the amino acid sequence as shown in
Figs. 4A and 4B (SEQ ID. NO: 20).

11. An isolated nucleic acid encoding the fusion polypeptide of claim 1.

12. A method for the delivery of a cytotoxic agent to a tumor cell comprising the administration of a fusion polypeptide comprising antibody variable light and heavy region sequences operatively linked to a β-lactamase, wherein the fusion polypeptide is reactive with a tumor cell antigen and the fusion polypeptide converts a prodrug to a cytotoxic drug, and the administration of an effective amount of the prodrug.

13. The method of claim 12, wherein the prodrug is derived from phenyleneamine mustard, doxorubicin, mitomycin C, paclitaxel, a vinca alkaloid, or melphalan.

14. The method of claim 12, wherein the tumor cell antigen is p97.

15. The method of claim 12, wherein the β-lactamase is *E. cloacae* P99 having one or more amino acid substitutions at residues 537-541.

16. The method of claim 15, wherein the amino acid substitutions are Thr Ser Phen Gly Asn (SEQ ID. NO: 2).

17. The method of claim 12, wherein the antibody variable light and heavy region sequences are separated by a spacer.

18. The method of claim 17, wherein the spacer is Gly Ser Thr Ser Gly Ser Gly Lys Pro Gly Ser Gly Glu Gly Ser Thr Lys Gly (SEQ ID. NO: 13).

FIG. 1

CCM

PDM

FIG. 3

FIG. 2

FIG. 7

FIG. 4A

```
AATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAACAATTCCCCTCTAGAAATAATTTTGTTAACTTTAAGAAGGAGATATACA
                                                                              L49 HEAVY CHAIN V

TATGAAATACCTGCTGCCGACCGCTGCTGCGGTCGCTGCTGGTCTGCTCCTGCTGCCAGCCGCGATGGCCGAGGTGCAGCTTCAGGAGTCAGG
   M  K  Y  L  L  P  T  A  A  A  G  L  L  L  L  A  A  Q  P  A  M  A  E  V  Q  L  Q  E  S  G
 9

ACCTAGCCTCCTGAAACCTTCTCAGACTCTGTCCCTCACCTGCTCTGTCACTGGTTACTGGAACTGGATCCG
 P  S  L  V  K  P  S  Q  T  L  S  L  T  C  S  V  T  G  D  S  I  T  S  G  Y  W  N  W  I  R
39

GAAGTTCCCAGGGAATAAACTTGAATATATGGGTTACATATGGGTATCATTACTACAATCCATCTCTCAAAAGTCGCATTTC
 K  F  P  G  N  K  L  E  Y  M  G  Y  I  S  D  S  G  I  T  Y  Y  N  P  S  L  K  S  R  I  S
69

CATCACTCGAGACACCATCCAAGAACAACTACCTCCAGTTGAATTTTGTGACTGCTGAGGACACAGCCACATATAACTGTGCAAGAAG
 I  T  R  D  T  S  K  N  Q  Y  Y  L  Q  L  N  F  V  T  A  E  D  T  A  T  Y  N  C  A  R  R
                                                                              218 LINKER

GACTCTGGCTACTACTGCTATGGACTATTGGGGTCAAGGAACCCTCTCACCGTCTCCTCAggctcggctccggctctggcgaacc
 T  L  A  T  Y  Y  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S  G  S  T  S  G  S  G  K  P
99                               L49 LIGHT CHAIN V

gggctctggcggaggctctaccagaggcGATTTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCAT
    G  S  G  E  G  S  T  K  G  D  F  V  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I
129

CTCTTGCAGGGCTAGTCAGAGCCTTGTACACAGTAATGGAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAGCT
 S  C  R  A  S  Q  S  L  V  H  S  N  G  N  T  Y  L  H  W  Y  L  Q  K  P  G  Q  S  P  K  L
159

CCTGATCTCTACAGAGTTCCAACCGATTTCTGGGGTCCCAGACAGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAG
 L  I  Y  R  V  S  N  R  F  S  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I  S
189

CAGAGTGGAGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAAAGTACACATGTTCCTCCGACGTTCGGTGGAGGCACCAAGCTGGAAAT
 R  V  E  A  E  D  L  G  V  Y  F  C  S  Q  S  T  H  V  P  P  T  F  G  G  G  T  K  L  E  I
219                                                           BETA-LACTAMASE

CAAACGGACGGTCAGAAAAACAGCTGGCGGTCGCGGAATACGCCAATTACCCGCTGATGAAAGCCCAATCTGTTCCAGGCAT
 K  R  T  P  V  S  E  K  Q  L  A  E  V  V  A  N  T  I  T  P  L  M  K  A  Q  S  V  P  G  M
249

GGCGGTGGCCGTTATTATCAGGGAAAACCGCACTATTACACATTTGGCAAGGCCGATATCGCGGCCAATAAACCCGTTACCCCTCAGAC
 A  V  A  V  I  Y  Q  G  K  P  H  Y  Y  T  F  G  K  A  D  I  A  A  N  K  P  V  T  P  Q  T
279
```
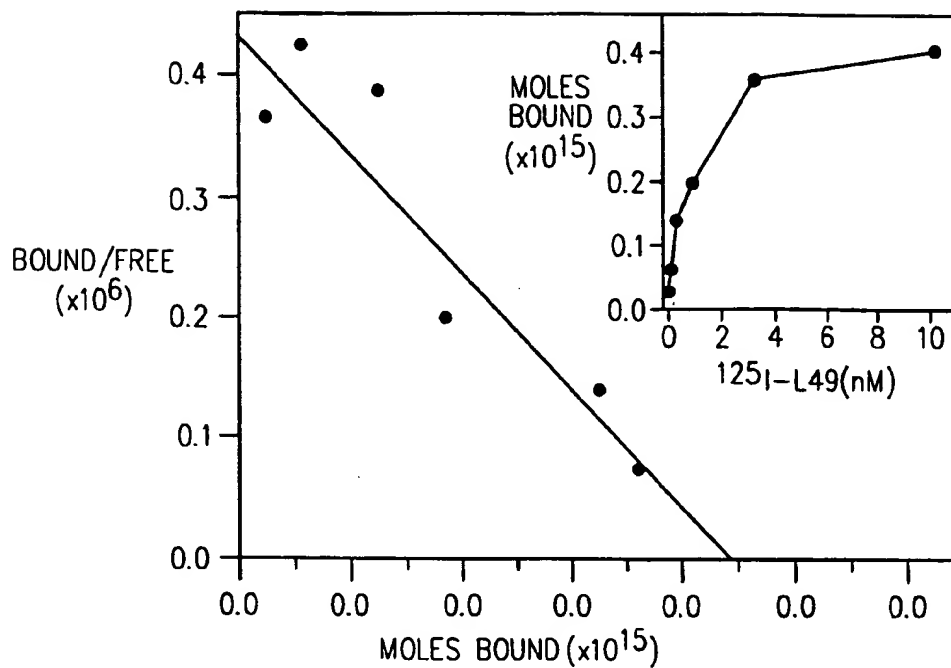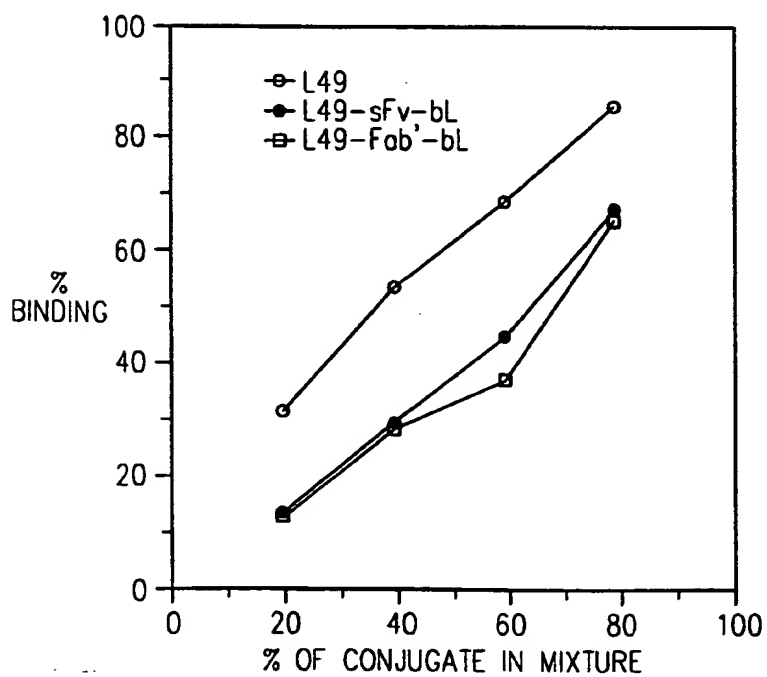
FIG. 4B

```
309 CCTGTTCGAGCTCGGGTTCTATAAGTAAAACCTTCACCGGGCGGTTTAGGTGGGGATGCCATTGCTCTCGCGGTGAAATTCGCTCGGGGTGAAGATGC
     L  F  E  L  G  S  I  S  K  T  F  T  G  V  L  G  G  D  A  I  A  R  G  E  I  S  L  D  D  A

339 GGTGACCAGAGATACTGGCCACAGCTGACGGGCAAGCAGTGGCAGGGCATTCGCATGCTCGATCTCGCTACACCGGTGGGGCCTGCCGGCGGCCTGCC
     V  T  R  Y  W  P  Q  L  T  G  K  Q  W  Q  G  I  R  M  L  D  L  A  T  Y  T  A  G  G  L  P

369 GCTACAGGTACCCGGATGAGGTCACGGATAACGCCTCCCTGCTGCGCTTTTATCAAAACTGGTACCCGCAGTGGAAGCCTGGCACAACGCG
     L  Q  V  P  D  E  V  T  D  N  A  S  L  L  R  F  Y  Q  N  W  Q  P  Q  W  K  P  G  T  T  R

399 TCTTTACGGCCAACGCCAGCCAGCATCGGTCTTTTGGTGCGCTGGCGGTCAAACCTTCTGGCATGCCCTATGAGCAGGCCATGACGACGCGGGT
     L  Y  A  N  A  S  I  G  L  F  G  A  L  A  V  K  P  S  G  M  P  Y  E  Q  A  M  T  T  R  V

429 CCTTAAGCCGCTCAAGCTGGACCATACCTGGATTAACGTGCCGAAAGCGGCGGAAGAGGCGCATTACGCCTGGGGCTATCGTGACGGGTAAAGC
     L  K  P  L  K  L  D  H  T  W  I  N  V  P  K  A  E  E  A  H  Y  A  W  G  Y  R  D  G  K  A

459 GGTGCGCGTTTCGCCCGGTATGCTGGATGCGCAAGCCTATGGCGTGAAAACCAACGTCACCGTCAGGATATGGCCAACTGGGTCATGGCCAAACAT
     V  R  V  S  P  G  M  L  D  A  Q  A  Y  G  V  K  T  N  V  Q  D  M  A  N  W  V  M  A  N  M

489 GGCGCCCGGAGAACGTTGCTGATGCCTCACTTAAGCAGGGGATCGCCCTACTGGCGCATCCGGGTCAATGTATCAGGG
     A  P  E  N  V  A  D  A  S  L  K  Q  G  I  A  L  A  Q  S  R  Y  W  R  I  G  S  M  Y  Q  G

                                                                              MUT. REGION
519 TCTGGGCTGGGAGATGCTCAACTGGCCGGTCGAG|Gacgagtttggtaat|GTAGCACTGGCGCCGTTGCCCGT
     L  G  W  E  M  L  N  W  P  V  E  A  N  T  V  V  E  T  S  F  G  N  V  A  L  A  P  L  P  V

549 GGCAGAAGTGAATCCACCGGCTCCCCCGGTTCCAAAGCGTCCAGCGTGGTCCACAAGACTGGCAGCACCGGCGGTTTCGGCAGCTACGTGGCCTT
     A  E  V  N  P  P  A  P  P  V  K  A  S  W  V  H  K  T  G  S  T  G  G  F  G  S  Y  V  A  F

579 TATTCCTGAAAAGCAGATCGGGATTGTGATGCTCGCCAATACCAGCTATCCGAATCCGGCACCGCGTTGAGGCGGCCGCATACCATATCCtcga
     I  P  E  K  Q  I  G  I  V  M  L  A  N  T  S  Y  P  N  P  A  R  V  E  A  A  Y  H  I  L  E

609 ggcgctacagtagactagtGAATTCGAGCT
     A  L  Q  *
```
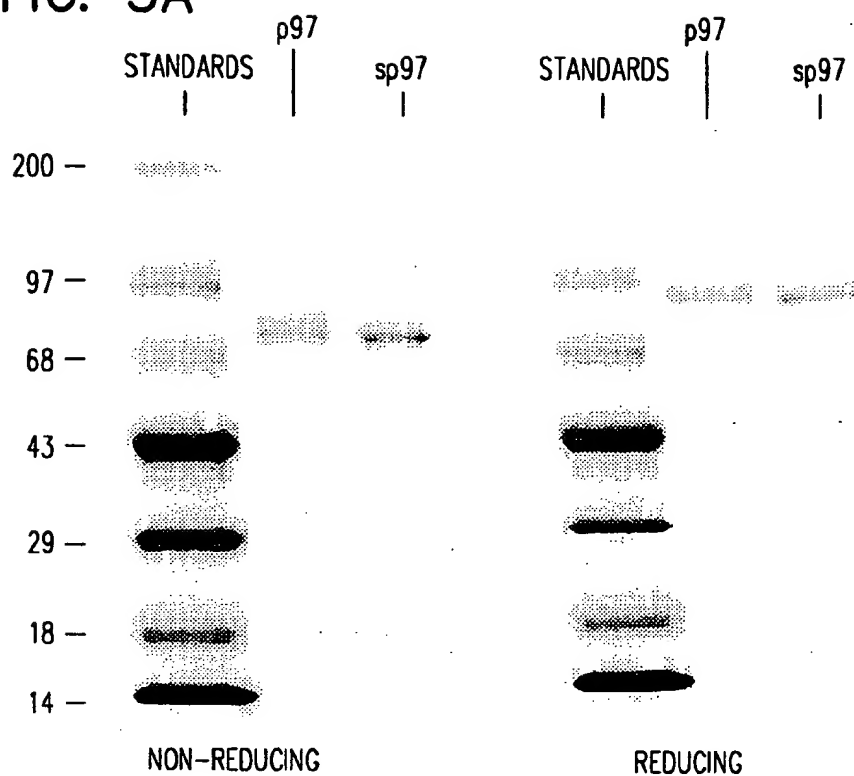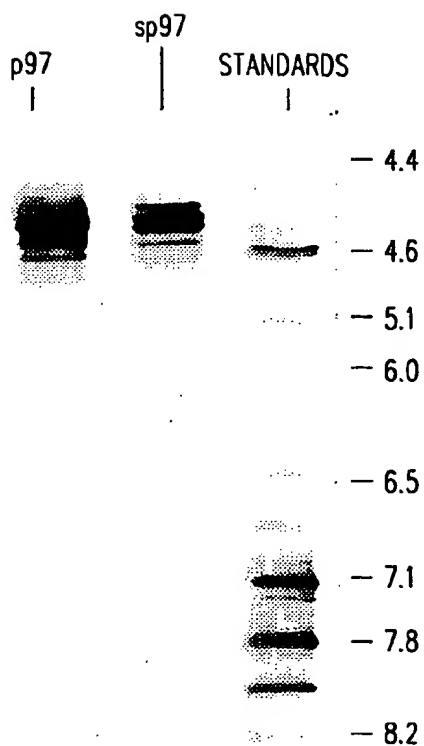
5/11

FIG. 5A

STANDARDS   p97   sp97   STANDARDS   p97   sp97

200 —

97 —

68 —

43 —

29 —

18 —

14 —

NON-REDUCING   REDUCING

FIG. 5B

p97   sp97   STANDARDS

— 4.4

— 4.6

— 5.1

— 6.0

— 6.5

— 7.1

— 7.8

— 8.2

SUBSTITUTE SHEET (RULE 26)

FIG. 6A

IPTG CONCENTRATION (μM)

| 100 | 50 | 25 | 12 | 6 | 3 | 1.6 | 0 |

FIG. 6B

FIG. 6C

FIG. 6D

FIG. 8

FIG. 9A

FIG. 9B

PERCENT INJECTED
DOSE/GRAM

■ 4 HOURS
□ 12 HOURS
▨ 24 HOURS

TUMOR　　LIVER　　SPLEEN　　KIDNEY　　BLOOD

FIG. 10A

TUMOR
VOLUME
(mm³)

L49-sFv-bL(1mg/kg), 12 HOURS LATER:
　　　　　　　　　　　　　　　CURES
△—CCM (75mg/kg)　　4/6
▲—CCM (125mg/kg)　　6/6
▽—CCM (175 mg/kg)　 6/6
●—UNTREATED　　　　0/6

TIME POST IMPLANT (DAYS)

FIG. 10B

L49-sFv-bL(1mg/kg), 24 HOURS LATER:
CURES
—△— CCM (150mg/kg)   3/6
—▲— CCM (225mg/kg)   6/6
—●— UNTREATED   0/6

FIG. 10C

L49-sFv-bL(1mg/kg), 24 HOURS LATER:
CURES
—△— CCM (75mg/kg)   3/6
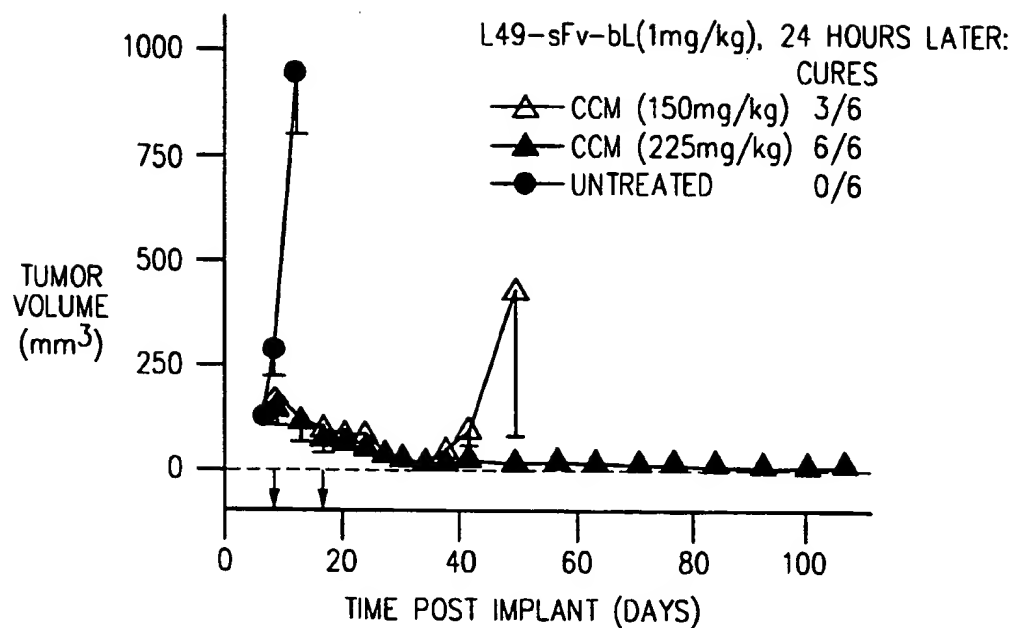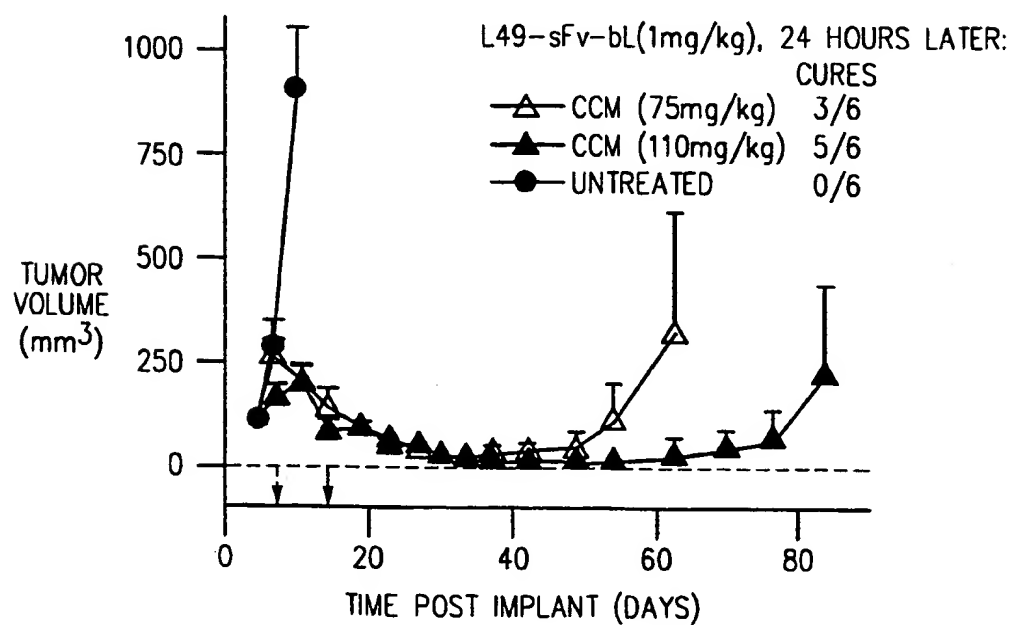—▲— CCM (110mg/kg)   5/6
—●— UNTREATED   0/6

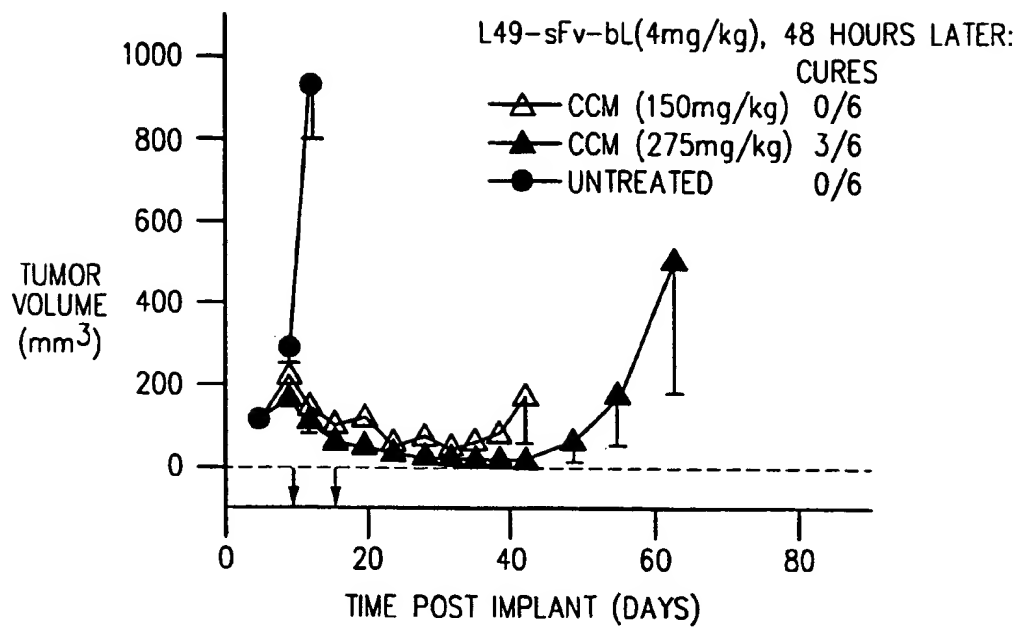## FIG. 10D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08840

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07K 16/00, 17/00; C07H 21/02, 21/04; A61K 39/395
US CL   :530/391.1, 391.5, 391.7; 536/23.5; 424/130.1, 133.1, 138.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   530/391.1, 391.5, 391.7; 536/23.5; 424/130.1, 133.1, 138.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE
search terms: lactamase, antibod?, fusion

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RODRIGUES, M.L. et al. Development of Humanized Disulfide-stabilized Anti-p185^{HER2} Fv-beta-Lactamase Fusion Protein for Activation of a Cephalosporin Doxorubicin Prodrug. Cancer Research. 01 January 1995, Vol. 55, pages 63-70, see abstract.	1-2, 6, 8, 12, 13, 17
X	KERR, D.E. et al. Regressions and Cures of Melanoma Xenografts following Treatment with Monoclonal antibody beta-Lactamase Conjugates in combination with Anticancer Prodrugs. Cancer Research. 15 August 1995, Vol. 55, pages 3558-3563, see abstract.	1-2, 6, 8-9, 12-14, 17

| ☒ | Further documents are listed in the continuation of Box C. | ☐ | See patent family annex. |

*   Special categories of cited documents

"A"   document defining the general state of the art which is not considered to be of particular relevance

"E"   earlier document published on or after the international filing date

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Date of the actual completion of the international search	Date of mailing of the international search report
13 JULY 1998	0 2 SEP 1998

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document. with indication. where appropriate. of the relevant passages	Relevant to claim No.
X	GOSHORN, S.C. et al. Genetic Construction, Expression, and Characterization of a Single chain Anti-Carcinoma Antibody Fused to beta-Lactamase. Cancer Research. 01 May 1993, Vol. 53, pages 2123-2127, see pages 2123-2124.	1-3, 6, 8, 11
X ------ Y	DE SUTTER, K. et al. A Bifunctional Murine::Human chimeric Antibody with one Antigen-Binding arm replaced by bacterial beta-Lactamase. Molecular Immunoogy, 1994, Vol. 31, pages 261-267, see abstract.	1, 11 -------- 12-13
X,P	SIEMERS, N.O. et al. Construction, Expression and Activities of L49-sFv-Beta-lactamse, a single-Chain Antibody Fusion Protein for Anticancer Prodrug Activation. Bioconjugate Chemistry. 1997. Vol. 8, pages 510-519, see abstract.	1-4, 6, 8-9, 11-12, 14, 17